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In order to produce a soluble AH-1.23 TCR incorporating a novel disulphide bond, TCR plasmids containing the  $\alpha$  chain BamHI and  $\beta$  chain BglII restriction sites were used as a framework as described in Example 4. The following primers were used:

5 | NdeI |  
5' -GGGAAGCTTACATATGAAGGAGGTGGAGCAGAATTCTGG-3'

5' -TACACGGCAGGATCCGGGTTCTGGATATT-3'

| BamHI |

10 | NdeI |  
5' -TTGGAATTCACATATGGCGTCATGCAGAACCAAGACAC-3'

5' -CCCAAGCTTAGTCTGCTCTACCCCAGGCCTCGGC-3'

| BglII |

15 AH-1.23 TCR  $\alpha$  and  $\beta$ -chain constructs were obtained by PCR cloning as follows. PCR reactions were performed using the primers as shown above, and templates containing the AH-1.23 TCR chains. The PCR products were restriction digested with the relevant restriction enzymes, and cloned into pGMT7 to obtain expression plasmids. The sequence of the plasmid inserts were confirmed by automated DNA sequencing. Figures 33a and 33b show the DNA sequence of the mutated  $\alpha$  and  $\beta$  chains of the AH-1.23 TCR respectively, and Figures 34a and 34b show the resulting amino acid sequences.

20 The respective TCR chains were expressed, co-refolded and purified as described in Example 5.

Figure 35 illustrates the elution of soluble AH-1.23 disulphide-linked TCR protein elution from a POROS 50HQ anion exchange column using a 0-500 mM NaCl gradient, as indicated by the dotted line. Figures 36 and 37 show the results of 30 reducing SDS-PAGE (Coomassie-stained) and non-reducing SDS-PAGE (Coomassie-stained) gels respectively of fractions from the column run illustrated by Figure 35. These gels clearly indicate the presence of a TCR heterodimer which is inter-chain disulphide linked. Figure 38 is the elution profile from a Superdex 75 HR gel filtration column of pooled fractions from the anion exchange column run illustrated in

Figure 35. The protein elutes as a single major peak, corresponding to the heterodimer.

5     *Example 8 – Production of soluble A6 TCRs containing a novel disulphide inter-chain bond at alternative positions within the immunoglobulin region of the constant domain*

The following experiments were carried out in order to investigate whether it was possible to form functional soluble TCRs which include a novel disulphide bond in the TCR immunoglobulin region at a position other than between threonine 48 of exon 1  
10    in TRAC\*01 and serine 57 of exon 1 in both TRBC1\*01 / TRBC2\*01.

For the mutating the A6 TCR  $\alpha$ -chain, the following primers were designed (the numbers in the primer names refer to the position of the amino acid residue to be mutated in exon 1 of TRAC\*01, mutated residues are shown in lower case):

15

T48→C Mutation

5'-CACAGACAAAtgTGTGCTAGACAT-3'

5'-ATGTCTAGCACACaTTGTCTGTG-3'

20    Y10→C Mutation

5'-CCCTGCCGTGTgCCAGCTGAGAG-3''

5'-CTCTCAGCTGGcACACGGCAGGG-3'

L12→C Mutation

25    5'-CCGTGTACCAAGtgcAGAGACTCTAAATC-3'

5'-GATTAGAGTCTCTgcaCTGGTACACGG-3'

S15→C Mutation

5'-CAGCTGAGAGACTgTAAATCCAGTGAC-3'

30    5'-GTCACTGGATTAcAGTCTCTCAGCTG-3'

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V22→C Mutation

5'-CAGTGACAAGTCTtgCTGCCTATTAC-3'

5'-GTGAATAGGCAGcaAGACTTGTCACTG-3'

5 Y43→C Mutation

5'-GATTCTGATGTGTgTATCACAGACAAAT-3'

5'-ATTGTCTGTGATAcACACATCAGAAC-3'

T45→C Mutation

10 5'-CTGATGTGTATATCtgtGACAAAActGTGC-3'

5'-GCACAGTTTGTcacaGATATACACATCAG-3'

L50→C Mutation

5'-AGACAAAActGTGtgtGACATGAGGTCT-3'

15 5'-AGACCTCATGTcacaCACAGTTTGTCT-3'

M52→C Mutation

5'-ACTGTGCTAGACtgtAGGTCTATGGAC-3'

5'-GTCCATAGACCTacaGTCTAGCACAGT-3'

20

S61→C Mutation

5'-CTTCAAGAGCAACtgtGTGCTGTGGCC-3'

5'-GGCACAGCACaGTTGCTCTGAAG-3'

25 For mutating the TCR A6  $\beta$ -chain, the following primers were designed (the numbers in the primer names refer to the position of the amino acid residue to be mutated in exon 1 of TRBC2\*01. Mutated residues are shown in lower case):

S57→C Mutation

30 5'-CAGTGGGGTtgtGCACAGACCC-3'

5'-GGGTCTGTGCaGACCCCACTG-3'

## V13→C Mutation

5'-CCGAGGTCGCTtgtTTTGAGGCCATCAG-3'

5'-CTGATGGCTCAAAacaAGCGACCTCGG-3'

5

## F14→C Mutation

5'-GGTCGCTGTGtgtGAGCCATCAGA-3'

5'-TCTGATGGCTCacaCACAGCGACC-3'

## 10 S17→C Mutation

5'-GTGTTGAGCCATgtGAAGCAGAGATC-3'

5'-GATCTCTGCTTCacATGGCTAAACAC-3'

## G55→C Mutation

## 15 5'-GAGGTGCACAGTtGtGTCAGCACAGAC-3'

5'-GTCTGTGCTGACaCaACTGTGCACCTC-3'

## D59→C Mutation

5'-GGGTCAGCACAtgCCCGCAGCCC-3'

## 20 5'-GGGCTGCGGGCaTGTGCTGACCC-3'

## L63→C Mutation

5'-CCCGCAGCCCtgCAAGGAGCAGC-3'

5'-GCTGCTCCTTGCaGGGCTGCGGG-3'

25

## S77→C Mutation

5'-AGATACGCTCTGtgtGCAGCCGCCT-3'

5'-AGGCGGCTGCaCAGAGCGTATCT-3'

## 30 R79→C Mutation

5'-CTCTGAGCAGCtgGCCTGAGGGTC-3'

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5'-GACCCTCAGGCaGCTGCTCAGAG-3'

E15→C Mutation

5' -GCTGTGTTtgtCCATCAGAA- 3'

5 5' -TTCTGATGGacaAAACACAGC- 3'

PCR mutagenesis,  $\alpha$  and  $\beta$  TCR construct amplification, ligation and plasmid purification was carried out as described in Example 1 using the appropriate combination of the above primers in order to produce soluble TCRs including novel disulphide inter-chain bonds between the following pairs of amino acids:

TCR $\alpha$ chain	TCR $\beta$ chain	$\alpha$ Primer used	$\beta$ Primer used
Thr 48	Ser 57	T48→C	S57→C
Thr 45	Ser 77	T45→C	S77→C
Ser 61	Ser 57	S61→C	S57→C
Leu 50	Ser 57	L50→C	S57→C
Tyr 10	Ser 17	Y10→C	S17→C
Ser 15	Val 13	S15→C	V13→C
Thr 45	Asp 59	T45→C	D59→C
Leu 12	Ser 17	L12→C	S17→C
Ser 61	Arg 79	S61→C	R79→C
Leu 12	Phe 14	L12→C	F14→C
Val 22	Phe 14	V22→C	F14→C
Met 52	Gly 55	M52→C	G55→C
Tyr 43	Leu 63	Y43→C	L63→C
Ser 15	Glu 15	S15→C	E15→C

Figures 39 to 58 show the DNA and amino acid sequences of the mutated A6 TCR chains amplified by the above primers. The codons encoding the mutated cysteines 15 are highlighted.

The respective TCR chains were expressed, co-refolded and purified as described in Example 5. Following purification on POROS 50HQ anion exchange column, the resulting proteins were run on SDS-Page gels in order to assess whether any correctly-refolded soluble TCR had been formed. These gels were also assessed to ascertain the 5 presence or absence of any disulphide-linked protein of the correct molecular weight in the purified material. TCRs under investigation containing the following novel disulphide inter-chain bonds failed to produce disulphide-linked protein of the correct molecular weight using this bacterial expression system and these were not further assessed. However, alternative prokaryotic or eukaryotic expression systems are 10 available.

TCR $\alpha$ chain	TCR $\beta$ chain
Ser 61	Ser 57
Leu 50	Ser 57
Ser 15	Val 13
Leu 12	Ser 17
Ser 61	Arg 79
Leu 12	Phe 14
Val 22	Phe 14
Tyr 43	Leu 63

Figures 59 to 64 respectively illustrate the elution of soluble TCRs containing novel disulphide interchain bonds between the following residues: Thr 48-Ser 57, Thr 45-Ser 15 77, Tyr 10-Ser 17, Thr 45-Asp 59, Met 52-Gly 55 and Ser 15-Glu 15 from a POROS 200HQ anion exchange column using a 0-500 mM NaCl gradient, as indicated by the dotted line. Figures 65 to 70 show the results of reducing SDS-PAGE (Coomassie-stained) and non-reducing SDS-PAGE (Coomassie-stained) gels respectively of fractions from the column runs illustrated by Figures 59 to 64. These gels clearly 20 indicate the presence of TCR heterodimers that are inter-chain disulphide linked.

Figures 71 to 76 are elution profiles from a Superdex 200 HR gel filtration column of pooled fractions from the anion exchange column runs illustrated in Figures 59 to 64.

A BIACore analysis of the binding of the TCRs to pMHC was carried out as described in Example 3. Figures 77- 82 are BIACore traces demonstrating the ability of the purified soluble TCRs to bind to HLA-A2 tax pMHC complexes.

5 Thr 48-Ser 57 had a  $K_d$  of 7.8  $\mu\text{M}$ , Thr 45-Ser 77 had a  $K_d$  of 12.7  $\mu\text{M}$ , Tyr 10-Ser 17 had a  $K_d$  of 34  $\mu\text{M}$ , Thr 45-Asp 59 had a  $K_d$  of 14.9  $\mu\text{M}$ , and Ser 15-Glu 15 had a  $K_d$  of 6.3  $\mu\text{M}$ . Met 52-Gly 55 was capable of binding to its native “target”, the HLA-A2 tax complex, although it also bound in a similar manner to an “irrelevant” target, the HLA-A2-NY-ESO complex (see Figure 81)

10

*Example 9 – X-ray crystallography of the disulphide-linked NY-ESO T cell receptor, specific for the NY-ESO-HLA-A2 complex.*

The NY-ESO dsTCR was cloned as described in Example 5, and expressed as follows.

15

The expression plasmids containing the mutated  $\alpha$ -chain and  $\beta$ -chain respectively were transformed separately into *E.coli* strain BL21 pLysS, and single ampicillin-resistant colonies were grown at 37°C in TYP (ampicillin 100 $\mu\text{g}/\text{ml}$ ) medium to  $\text{OD}_{600}$  of 0.7 before inducing protein expression with 0.5mM IPTG. Cells were harvested 18

20 hours post-induction by centrifugation for 30 minutes at 4000rpm in a Beckman J-6B. Cell pellets were resuspended in lysis buffer containing 10mM Tris-HCl pH 8.1, 10 mM MgCl<sub>2</sub>, 150 mM NaCl, 2 mM DTT, 10% glycerol. For every 1 L of bacterial culture 100  $\mu\text{l}$  of lysozyme (20 mg/ml) and 100  $\mu\text{l}$  of Dnase I (20  $\mu\text{g}/\text{ml}$ ) were added. After incubation on ice for 30 minutes, the bacterial suspension was sonicated in 1

25 minute bursts for a total of 10 minutes using a Milsonix XL2020 sonicator with a standard 12mm diameter probe. Inclusion body pellets were recovered by centrifugation for 30 minutes at 13000rpm in a Beckman J2-21 centrifuge (4 °C).

Three washes were then carried out in Triton wash buffer (50mM Tris-HCl pH 8.1, 0.5% Triton-X100, 100mM NaCl, 10mM NaEDTA, 0.1% (w/v), 2mM DTT) to

30 remove cell debris and membrane components. Each time, the inclusion body pellet was homogenised in Triton wash buffer before being pelleted by centrifugation for 15 minutes at 13000rpm in a Beckman J2-21. Detergent and salt was then removed by a

similar wash in Resuspension buffer (50mM Tris-HCl pH 8.1 100mM NaCl, 10mM NaEDTA, 0.1% (w/v) NaAzide, 2mM DTT). Finally, the inclusion bodies were solubilised in 6 M guanidine buffer (6 M Guanidine-hydrochloride, 50mM Tris pH 8.1, 100mM NaCl, 10mM EDTA, 10mM DTT), divided into 120 mg aliquots and 5 frozen at -70°C. Inclusion bodies were quantitated by solubilising with 6M guanidine-HCl and measurement with a Bradford dye-binding assay (PerBio).

Approximately 60mg (i.e. 2.4  $\mu$ mole) of frozen solubilised alpha chain was mixed with 30 mg (i.e. 1.2  $\mu$ mole) of frozen solubilised beta chain. The TCR mixture was 10 diluted to a final volume of 18ml with 6 M guanidine buffer and heated to 37 °C for 30 min to ensure complete chain denaturation. The guanidine solution containing fully reduced and denatured TCR chains was then mixed into 1 litre of cold refolding buffer (100mM Tris pH 8.1, 400mM L-Arginine-HCl, 2mM EDTA, 6.6 mM 2-mercaptopethanol, 3.7 mM Cystamine, 5M urea) with stirring. The solution was 15 left for 5 hrs in the cold room (5°C  $\pm$  3°C) to allow refolding to take place. The refold was then dialysed against 12 litres of water for 18-20 hours, followed by 12 litres of 10mM Tris pH 8.1 for 18-20 hours (5°C  $\pm$  3°C). Spectrapor 1 (Spectrum Laboratories product no. 132670) dialysis membrane that has a molecular weight cut off of 6-8000kDa was used for this dialysis process. The dialysed protein was filtered through 20 0.45  $\mu$ m pore size filters (Schleicher and Schuell, Ref. number, 10 404012) fitted to a Nalgene filtration unit.

The refolded NY-ESO TCR was separated from degradation products and impurities by loading the dialysed refold onto a POROS 50HQ (Applied Biosystems) anion 25 exchange column using an ÄKTA purifier (Amersham Biotech). A POROS 50 HQ column was pre-equilibrated with 10 column volumes of buffer A (10 mM Tris pH 8.1) prior to loading with protein. The bound protein was eluted with a gradient of 0-500mM NaCl over 7 column volumes. Peak fractions (1 ml) were analysed on denaturing SDS-PAGE using reducing and non-reducing sample buffer. Peak 30 fractions containing the heterodimeric alpha-beta complex were further purified using a Superdex 75HR gel filtration column pre-equilibrated in 25 mM MES pH 6.5. The protein peak eluting at a relative molecular weight of approximately 50 kDa was

pooled, concentrated to 42 mg/ml in Ultrafree centrifugal concentrators (Millipore, part number UFV2BGC40) and stored at -80 °C.

Crystallisation of NY-ESO TCR was performed by hanging drop technique at 18 °C  
5 using 1 µl of protein solution (8.4 mg/ml) in 5 mM Mes pH 6.5 mixed with an equivalent volume of crystallisation buffer. Crystals appeared under several different conditions using Crystal Screen buffers (Hampton Research). Single cubic crystals (< 100 µm) were grown in 30 % PEG 4000, 0.1 M Na Citrate pH 5.6, 0.2 M ammonium acetate buffer and used for structure determination.

10

Crystals of the NY-ESO TCR were flash-frozen and tested for diffraction in the X-ray beam of the Daresbury synchrotron. The crystals diffracted to 0.25nm (2.5Å) resolution. One data set was collected and processed to give a 98.6% complete set of amplitudes that were reasonable to around 0.27 nm (2.7Å), but usable up to 0.25 nm  
15 (2.5Å). The merging R-factor, i.e. the agreement between multiple measurements of crystallographically equivalent reflections, was 10.8% for all the data. This is marginal at the highest resolution shell. The space group was P2<sub>1</sub>, with cell dimensions a=4.25 nm (42.5Å), b=5.95 nm (59.5Å), c=8.17 nm (81.7 Å), β=91.5°. The cell dimensions and symmetry meant there were two copies in the cell. The  
20 asymmetric unit, au or the minimum volume that needs to be studied, has only 1 molecule, and the other molecule in the cell is generated by the 2<sub>1</sub> symmetry operation. The positioning of the molecule in the au is arbitrary in the y-direction. As long as it is in the correct position in the x-z plane, it can be translated at will in the y-direction. This is referred to as a free parameter, in this 'polar' space group.

25

The PDB data base has only one entry containing an A/B heterodimeric TCR, 1BD2. This entry also has co-ordinates of the HLA-cognate peptide in complex with the TCR. The TCR chain B was the same in NY-ESO, but chain A had small differences in the C-domain and significant differences in the N-domain. Using the 1BD2 A/B  
30 model for molecular replacement, MR, gave an incorrect solution, as shown by extensive overlap with symmetry equivalent molecules. Using the B chain alone gave a better solution, which did not have significant clashes with neighbours. The

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correlation coefficient was 49%, the crystallographic R-factor 50%, and the nearest approach (centre-of-gravity to c-o-g) was 0.49 nm (49Å). The rotation and translation operation needed to transform the starting chain B model to the MR equivalent, was applied to chain A. The hybrid MR solution thus generated, packed well in the cell,  
5 with minimal clashes.

Electron density maps generally agreed with the model, and allowed its adjustment to match the sequence of the NY-ESO TCR. But the starting model had many gaps, specifically missing side-chains, that are characteristic of poorly ordered portions of  
10 the model. Many of the hair-pin loops in between strands had very low density, and were difficult to model. The crystallographic R-factor of the model is 30%. The R-factor is a residual, i.e. it is the difference between the calculated and observed amplitudes.

15 As Figures 83a and 83b demonstrate, the input sequence from 1BD2 do not match up with the density very well. Changing the model for Cys at positions 164 in chain A, and 174 in chain B, followed by further refinement, showed clearly that this sequence assignment is much better fitted to the density. But the differences in terms of size of the side chain are minimal, so there was little perturbation in the model. The electron  
20 density in that region is little changed.

The most important aspect of this work is that the new TCR is very similar in structure to the published model (1BD2). The comparison could include all of the TCR, the constant domains, or the small part near the mutation point.

25

The r.m.s deviation values are listed in the table below. The comparison of structures is shown in Figure 84.

	Chain A Complete	Chain B Complete	Chain A Constant	Chain B Constant	Short Stretch
r.m.s Displacement	2.831	1.285	1.658	1.098	0.613
Mean Displacement	2.178	1.001	1.235	0.833	0.546
Max Displacement	9.885	6.209	6.830	4.490	1.330

(All units are in Å)

The short stretch refers to the single strand from Chain A (A157 to A169) and the single strand from Chain B (B170 to B183) that are now joined by the disulphide bridge. The deviations were calculated for only the main chain atoms.

These results show that the introduction of the disulphide bond has minimal effect on the local structure of the TCR around the bond. Some larger effects are observed when comparing the TCR to the published structure (1BD2) of the A6 TCR, but the increase in RMS displacement is largely due to differences in loop conformations (see Figure 84). These loops do not form part of the core structure of the TCR, which is formed by a series of  $\beta$ -sheets which form a characteristic Ig fold. The RMS deviation for the whole  $\alpha$ -chain is particularly large because of the difference in the sequence of the variable domains between the A6 (1BD2) and the NY-ESO TCRs. However, the A6 and NY-ESO TCRs have the same variable  $\beta$ -domain and the RMS deviations for the whole  $\beta$ -chain show that the structure of this variable domain is also maintained in the TCR with the new disulphide bond. These data therefore indicate that the core structure of the TCR is maintained in the crystal structure of the TCR with the new disulphide bond.

20

*Example 10 – Production of soluble NY-ESO TCRs containing a novel disulphide inter-chain bond, and C-terminal  $\beta$  chain tagging sites.*

In order to produce a soluble NY-ESO TCR incorporating a novel disulphide bond, A6 TCR plasmids containing the  $\alpha$  chain BamHI and  $\beta$  chain BglIII restriction sites were used as frameworks as described in Example 4.

NY-ESO TCR  $\beta$ -chain constructs were obtained by PCR cloning as follows. PCR reactions were performed using the primers as shown below, and templates containing the NY-ESO TCR chains.

5

| NdeI |  
Fwd5' -GGAGATATACATATGGGTGTCACTCAGAAC- 3'  
Rev5' -CCACCGGATCCGTCTGCTCTACCCCAGGC- 3'  
| BamHI |

10

The PCR products were restriction digested with the relevant restriction enzymes, and cloned into pGMT7 containing the biotin recognition sequence to obtain expression plasmids. The sequence of the plasmid inserts were confirmed by automated DNA sequencing. Figure 85a shows the DNA sequence of the  $\beta$  chain of the NY-ESO TCR 15 incorporating the biotin recognition site, and Figure 85b shows the resulting amino acid sequence.

The  $\alpha$  chain construct was produced as described in Example 5. The respective TCR chains were expressed, co-refolded and purified as described in Example 5.

20

In order to produce a soluble NY-ESO TCR containing a non-native disulphide inter-chain bond and a hexa-histidine tag on the C- terminus of the  $\beta$  chain, the same primers and NY-ESO template were used as above. The PCR products were restriction digested with the relevant restriction enzymes, and cloned into pGMT7 25 containing the hexa-histidine sequence to obtain expression plasmids. Figure 86a shows the DNA sequence of the  $\beta$  chain of the NY-ESO TCR incorporating the hexa-histidine tag, and Figure 86b shows the resulting amino acid sequence.

Figure 87 illustrates the elution of soluble NY-ESO TCR containing a novel 30 disulphide bond and the biotin recognition sequence from a POROS 50HQ anion exchange column using a 0-500 mM NaCl gradient, as indicated by the dotted line. Figure 88 illustrates the elution of soluble NY-ESO TCR containing a novel

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disulphide bond and the hexa-histidine tag from a POROS 50HQ anion exchange columns using a 0-500 mM NaCl gradient, as indicated by the dotted line.

Figures 89 and 90 are protein elution profiles from gel filtration chromatography of 5 pooled fractions from the NY-ESO-biotin and NY-ESO-hexa-histidine tagged anion exchange column runs illustrated by Figures 87 and 88 respectively. The protein elutes as a single major peak, corresponding to the TCR heterodimer.

A BIACore analysis of sTCR binding to pMHC was carried out as described in 10 Example 3. The NY-ESO-biotin TCR had a Kd of 7.5  $\mu$ M, The NY-ESO-hexa-histidine tagged TCR had a Kd of 9.6  $\mu$ M

*Example 11 – Cell staining using fluorescent labelled tetramers of soluble NY-ESO TCR containing a novel disulphide inter-chain bond.*

15

#### *TCR Tetramer preparation*

The NY-ESO soluble TCRs containing a novel disulphide bond and a biotin recognition sequence prepared as in Example 10 were utilised to form the soluble TCR tetramers using required for cell staining. 2.5 ml of purified soluble TCR solution (~ 20 0.2 mg/ml) was buffer exchanged into biotinylation reaction buffer (50 mM Tris pH 8.0, 10 mM MgCl<sub>2</sub>) using a PD-10 column (Pharmacia). The eluate (3.5 ml) was concentrated to 1 ml using a centricon concentrator (Amicon) with a 10 kDa molecular weight cut-off. This was made up to 10mM with ATP added from stock (0.1 g/ml adjusted to pH 7.0). A volume of a cocktail of protease inhibitors was then added 25 (protease inhibitor cocktail Set 1, Calbiochem Biochemicals ), sufficient to give a final protease cocktail concentration of 1/100<sup>th</sup> of the stock solution as supplied, followed by 1 mM biotin (added from 0.2M stock) and 20  $\mu$ g/ml enzyme (from 0.5 mg/ml stock). The mixture was then incubated overnight at room temperature. Excess biotin was removed from the solution by size exclusion chromatography on a S75 HR 30 column. The level of biotinylation present on the NY-ESO TCR was determined via a size exclusion HPLC-based method as follows. A 50ul aliquot of the biotinylated NY-ESO TCR (2mg/ml) was incubated with 50ul of streptavidin coated agarose beads

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(Sigma) for 1 hour. The beads were then spun down, and 50  $\mu$ l of the unbound sample was run on a TSK 2000 SW column (Tosoohaas) using a 0.5ml/min flowrate (200mM Phosphate Buffer pH 7.0) over 30 minutes. The presence of the biotinylated NY-ESO TCR was detected by a UV spectrometer at both 214nm and 280nm. The biotinylated 5 NY-ESO was run against a non-biotinylated NY-ESO TCR control. The percentage of biotinylation was calculated by subtracting the peak-area of the biotinylated protein from that of the non-biotinylated protein.

Tetramerisation of the biotinylated soluble TCR was achieved using neutravidin-10 phycoerythrin conjugate (Cambridge Biosciences, UK). The concentration of biotinylated soluble TCR was measured using a Coomassie protein assay (Pierce), and a ratio of soluble TCR 0.8 mg/mg neutravidin-phycoerythrin conjugate was calculated to achieve saturation of the neutravidin-PE by biotinylated TCR at a ratio of 1:4. 15 19.5  $\mu$ l of a 6.15mg/ml biotinylated NY-ESO soluble TCR solution in phosphate buffered saline (PBS) was added slowly to 150  $\mu$ l of a 1mg/ml neutravidin-PE soluble over ice with gentle agitation. 100.5  $\mu$ l of PBS was then added to this solution to provide a final NY- ESO TCR tetramer concentration of 1 mg/ml.

*Staining Protocol*

20 Four aliquots of  $0.3 \times 10^6$  HLA-A2 positive EBV transformed B cell line (PP LCL) in 0.5ml of PBS were incubated with varying concentrations ( $0$ ,  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  M) of HLA-A2 NYESO peptide (SLLMWITQC) for 2 h at 37°C. These PP LCL cells were then washed twice in Hanks buffered Saline solution (HBSS) (Gibco, UK).

25 Each of the four aliquots were divided equally and stained with biotinylated NY-ESO disulphide linked TCR freshly tetramerised with neutravidin-phycoerythrin. Cells were incubated with either 5 or 10  $\mu$ g of phycoerythrin labelled tetrameric dsTCR complexes on ice for 30 minutes and washed with HBSS. Cells were washed again, re-suspended in HBSS and analysed by FACS Vantage. 25,000 events were collected 30 and data analysed using WinMIDI software.

*Results*

Figures 91a-h illustrate as histograms the FACS Vantage data generated for each of the samples prepared as described above. The following table lists the percentage of positively stained cells observed for each of the samples:

5

Sample	Positive stained Cells (%)
0 NY-ESO peptide, 5 $\mu$ g TCR	0.75
10 <sup>-4</sup> M NY-ESO peptide, 5 $\mu$ g TCR	84.39
10 <sup>-5</sup> M NY-ESO peptide, 5 $\mu$ g TCR	35.29
10 <sup>-6</sup> M NY-ESO peptide, 5 $\mu$ g TCR	7.98
0 NY-ESO peptide, 10 $\mu$ g TCR	0.94
10 <sup>-4</sup> M NY-ESO peptide, 10 $\mu$ g TCR	88.51
10 <sup>-5</sup> M NY-ESO peptide, 10 $\mu$ g TCR	8.25
10 <sup>-6</sup> M NY-ESO peptide, 10 $\mu$ g TCR	3.45

These data clearly indicate that the proportion of the cells labelled by the NY-ESO TCR tetramers increases in a manner correlated to the concentration of the peptide (SLLMWITQC) in which they had been incubated. Therefore, these NY-ESO TCR 10 tetramers are moieties suitable for specific cell labelling based on the expression of the HLA-A2 NY-ESO complex.

In the present example, a fluorescent conjugated NY-ESO TCR tetramer has been used. However, similar levels of cell binding would be expected if this label were 15 replaced by a suitable therapeutic moiety.

*Example 12 – Production of soluble A6 TCR with a novel disulphide bond incorporating the C $\beta$ 1 constant region.*

20 All of the previous examples describe the production of soluble TCRs with a novel disulphide bond incorporating the C $\beta$ 2 constant region. The present example

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demonstrates that soluble TCRs incorporating the C $\beta$ 1 constant region can be produced successfully.

*Design of primers for PCR stitching of A6 TCR  $\beta$ -chain V-domain to C $\beta$ 1.*

5 For PCR construct of A6 TCR  $\beta$ -chain V-domain, the following primers were designed:

5'-GGAGATATACATATGAACGCTGGTGTCACT-3'

5'-CCTTGTTCAGGTCTCTGTGACCGTGAG-3'

10

For PCR construct of C $\beta$ 1, the following primers were designed:

5'-CTCACGGTCACAGAGGACCTGAACAAAGG-3'

5'-CCCAAGCTTAGTCTGCTCTACCCAGGCCTCGGC-3'

15

Beta VTCR construct and C $\beta$ 1 construct were separately amplified using standard PCR technology. They were connected to each other using a stitching PCR. Plasmid DNA was purified on a Qiagen mini-prep column according to the manufacturer's instructions and the sequence was verified by automated sequencing at the sequencing facility of Department of Biochemistry, Oxford University. The sequence for 20 A6+C $\beta$ 1 is shown in Figure 92.

Consequently, the A6+C $\beta$ 1 chain was paired to A6 alpha TCR by inter-chain disulphide bond after introducing cysteine in C-domain of both chains.

25

The soluble TCR was expressed and refolded as described in Example 2.

*Purification of refolded soluble TCR:*

30 sTCR was separated from degradation products and impurities by loading the dialysed refold onto a POROS 50HQ anion exchange column and eluting bound protein with a gradient of 0-500mM NaCl over 50 column volumes using an Akta purifier

(Pharmacia) as in Figure 93. Peak fractions were stored at 4°C and analysed by Coomassie-stained SDS-PAGE (Figure 94) before being pooled and concentrated. Finally, the sTCR was purified and characterised using a Superdex 200HR gel filtration column (Figure 95) pre-equilibrated in HBS-EP buffer (10 mM HEPES pH 5 7.4, 150 mM NaCl, 3.5 mM EDTA, 0.05% nonidet p40). The peak eluting at a relative molecular weight of approximately 50 kDa was pooled and concentrated prior to characterisation by BIACore surface plasmon resonance analysis.

10 A BIACore analysis of the binding of the disulphide-linked A6 TCR to pMHC was carried out as described in Example 3. Figure 96 shows BIACore analysis of the specific binding of disulphide-linked A6 soluble TCR to its cognate pMHC.

15 The soluble A6 TCR with a novel disulphide bond incorporating the C $\beta$ 1 constant region had a  $K_d$  of  $2.42 \pm 0.55 \mu\text{M}$  for its cognate pMHC. This value is very similar to the  $K_d$  of  $1.8 \mu\text{M}$  determined for the soluble A6 TCR with a novel disulphide bond incorporating the C $\beta$ 2 constant region as determined in Example 3.

*Example 13 – Production of soluble A6 TCR with a novel disulphide bond incorporating the “free” cysteine in the  $\beta$  chain*

20 The  $\beta$  chain constant regions of TCRs include a cysteine residue (residue 75 in exon 1 of TRBC1\*01 and TRBC2\*01) which is not involved in either inter-chain or intra-chain disulphide bond formation. All of the previous examples describe the production of soluble TCRs with a novel disulphide bond in which this “free” cysteine 25 has been mutated to alanine in order to avoid the possible formation of any “inappropriate” disulphide bonds which could result in a reduced yield of functional TCR. The present example demonstrates that soluble TCRs incorporating this “free” cysteine can be produced.

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*Design of primers and mutagenesis of TCR  $\beta$  chain*

For mutating TCR  $\beta$ -chain alanine (residue 75 in exon 1 of TRBC1\*01 and TRBC2\*01) to cysteine, the following primers were designed (mutation shown in lower case):

5

5'-T GAC TCC AGA TAC tgT CTG AGC AGC CG  
5'-CG GCT GCT CAG Aca GTA TCT GGA GTC A

PCR mutagenesis, expression and refolding of the soluble TCR was carried out as  
10 described in Example 2.

*Purification of refolded soluble TCR:*

sTCR was separated from degradation products and impurities by loading the dialysed refold onto a POROS 50HQ anion exchange column and eluting bound protein with a  
15 gradient of 0-500mM NaCl over 50 column volumes using an Akta purifier (Pharmacia) as in Figure 98. Peak fractions were stored at 4°C and analysed by Coomassie-stained SDS-PAGE (Figure 99) before being pooled and concentrated. Finally, the sTCR was purified and characterised using a Superdex 200HR gel  
filtration column (Figure 100) pre-equilibrated in HBS-EP buffer (10 mM HEPES pH  
20 7.4, 150 mM NaCl, 3.5 mM EDTA, 0.05% nonidet p40). The peak eluting at a relative molecular weight of approximately 50 kDa was pooled and concentrated prior to characterisation by BIACore surface plasmon resonance analysis.

A BIACore analysis of the binding of the disulphide-linked A6 TCR to pMHC was  
25 carried out as described in Example 3. Figure 101 shows BIACore analysis of the specific binding of disulphide-linked A6 soluble TCR to its cognate pMHC.

The soluble A6 TCR with a novel disulphide bond incorporating the “free” cysteine in the  $\beta$  chain had a  $K_d$  of  $21.39 \pm 3.55 \mu\text{M}$  for its cognate pMHC.

30

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*Example 14 – Production of soluble A6 TCR with a novel disulphide bond wherein “free” cysteine in the  $\beta$  chain is mutated to serine.*

The present example demonstrates that soluble TCRs with a novel disulphide bond in  
5 which the “free” cysteine in the  $\beta$  chain (residue 75 in exon 1 of TRBC1\*01 and TRBC2\*01) is mutated to serine can be successfully produced.

*Design of primers and mutagenesis of TCR  $\beta$  chain*

For mutating TCR  $\beta$ -chain alanine that had previously been substituted for the native  
10 cysteine (residue 75 in exon 1 of TRBC1\*01 and TRBC2\*01) to serine, the following  
primers were designed (mutation shown in lower case):

5'-T GAC TCC AGA TAC tCT CTG AGC AGC CG  
5'-CG GCT GCT CAG AGa GTA TCT GGA GTC A

15

PCR mutagenesis (resulting in a mutated beta chain as shown in Figure 102),  
expression and refolding of soluble TCR was carried out as described in Example 2.

*Purification of refolded soluble TCR:*

20 sTCR was separated from degradation products and impurities by loading the dialysed  
refold onto a POROS 50HQ anion exchange column and eluting bound protein with a  
gradient of 0-500mM NaCl over 50 column volumes using an Akta purifier  
(Pharmacia) as shown in Figure 103. Peak fractions were stored at 4°C and analysed  
by Coomassie-stained SDS-PAGE (Figure 104) before being pooled and concentrated.  
25 Finally, the sTCR was purified and characterised using a Superdex 200HR gel  
filtration column (Figure 105) pre-equilibrated in HBS-EP buffer (10 mM HEPES pH  
7.4, 150 mM NaCl, 3.5 mM EDTA, 0.05% nonidet p40). The peak eluting at a relative  
molecular weight of approximately 50 kDa was pooled and concentrated prior to  
characterisation by BIACore surface plasmon resonance analysis.

30

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A BIACore analysis of the binding of the disulphide-linked A6 TCR to pMHC was carried out as described in Example 3. Figure 106 shows BIACore analysis of the specific binding of disulphide-linked A6 soluble TCR to its cognate pMHC.

5 The soluble A6 TCR with a novel disulphide bond in which the “free” cysteine in the  $\beta$  chain was mutated to serine had a  $K_d$  of  $2.98 \pm 0.27 \mu\text{M}$  for its cognate pMHC. This value is very similar to the  $K_d$  of  $1.8 \mu\text{M}$  determined for the soluble A6 TCR with a novel disulphide bond in which the “free” cysteine in the  $\beta$  chain was mutated to alanine as determined in Example 3.

10

*Example 15 – Cloning of NY-ESO TCR  $\alpha$  and  $\beta$  chains containing a novel disulphide bond into yeast expression vectors*

15 NY-ESO TCR  $\alpha$  and  $\beta$  chains were fused to the C-terminus of the pre-pro mating factor alpha sequence from *Saccharomyces cerevisiae* and cloned into yeast expression vectors pYX122 and pYX112 respectively (see Figures 107 and 108).

20 The following primers were designed to PCR amplify pre-pro mating factor alpha sequence from *S. cerevisiae* strain SEY6210 (Robinson *et al.* (1991), *Mol Cell Biol.* 11(12):5813-24) for fusing to the TCR  $\alpha$  chain.

5' -TCT GAA TTC ATG AGA TTT CCT TCA ATT TTT AC-3'  
5' -TCA CCT CCT GGG CTT CAG CCT CTC TTT TAT C -3'

25 The following primers were designed to PCR amplify pre-pro mating factor alpha sequence from *S. cerevisiae* strain SEY6210 for fusing to the TCR  $\beta$  chain.

5' -TCT GAA TTC ATG AGA TTT CCT TCA ATT TTT AC-3'  
5' -GTG TCT CGA GTT AGT CTG CTC TAC CCC AGG C-3'

30

Yeast DNA was prepared by re-suspending a colony of *S. cerevisiae* strain SEY6210 in  $30\mu\text{l}$  of 0.25% SDS in water and heating for 3 minutes at  $90^\circ\text{C}$ . The pre-pro mating

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factor alpha sequences for fusing to the TCR  $\alpha$  and  $\beta$  chains were generated by PCR amplifying 0.25  $\mu$ l of yeast DNA with the respective primer pairs mentioned above using the following PCR conditions. 12.5 pmoles of each primer was mixed with 200  $\mu$ M dNTP, 5  $\mu$ l of 10x Pfu buffer and 1.25 units of Pfu polymerase (Stratagene) in a final volume of 50  $\mu$ l. After an initial denaturation step of 30 seconds at 92°C, the reaction mixture was subjected to 30 rounds of denaturation (92°C, 30 sec.), annealing (46.9°C, 60 sec.), and elongation (72°C, 2 min.) in a Hybaid PCR express PCR machine.

10 The following primers were designed to PCR amplify the TCR  $\alpha$  chain to be fused to the pre-pro mating factor alpha sequence mentioned above.

5' -GGC TGA AGC CCA GGA GGT GAC ACA GAT TCC-3'  
5' -CTC CTC TCG AGT TAG GAA CTT TCT GGG CTG GG-3'

15 The following primers were designed to PCR amplify the TCR  $\beta$  chain to be fused to the pre-pro mating factor alpha sequence mentioned above.

5' -GGC TGA AGC CGG CGT CAC TCA GAC CCC AAA AT-3'  
20 5' -GTG TCT CGA GTT AGT CTG CTC TAC CCC AGG C-3'

25 The PCR conditions for amplifying the TCR  $\alpha$  and  $\beta$  chains were the same as mentioned above except for the following changes: the DNA template used for amplifying the TCR  $\alpha$  and  $\beta$  chains were the NY-ESO TCR  $\alpha$  and  $\beta$  chains respectively (as prepared in Example 5); and the annealing temperature used was 60.1°C.

30 The PCR products were then used in a PCR stitching reaction utilising the complementary overlapping sequences introduced into the initial PCR products to create a full length chimeric gene. The resulting PCR products were digested with the restriction enzymes EcoR I and Xho I and cloned into either pYX122 or pYX112 digested with the same enzymes. The resulting plasmids were purified on a Qiagen™

mini-prep column according to the manufacturer's instructions, and the sequences verified by automated sequencing at the sequencing facility of Genetics Ltd, Queensway, New Milton, Hampshire, United Kingdom. Figures 109 and 110 show the DNA and protein sequences of the cloned chimeric products.

5

*Example 16 – Expression of soluble NY-ESO TCR containing a novel disulphide bond in yeast*

The yeast expression plasmids containing the TCR  $\alpha$  and  $\beta$  chains respectively 10 produced as described in Example 15 were co-transformed into *S. cerevisiae* strain SEY6210 using the protocol by Agatep *et al.* (1998) (Technical Tips Online (<http://tto.trends.com>) 1:51:P01525). A single colony growing on synthetic dropout (SD) agar containing Histidine and Uracil (Qbiogene, Illkirch, France) was cultured overnight at 30°C in 10ml SD media containing Histidine and Uracil. The overnight 15 culture was sub-cultured 1:10 in 10ml of the fresh SD media containing Histidine and Uracil and grown for 4 hours at 30°C. The culture was centrifuged for 5 minutes at 3800rpm in a Heraeus Megafuge 2.0R (Kendro Laboratory Products Ltd, Bishop's Stortford, Hertfordshire, UK) and the supernatant harvested. 5 $\mu$ l StratClean Resin (Stratagene) was mixed with the supernatant and kept rotating in a blood wheel at 4°C 20 overnight. The StratClean resin was spun down at 3800rpm in a Heraeus Megafuge 2.0R and the media discarded. 25 $\mu$ l of reducing sample buffer (950 $\mu$ l of Laemmli sample buffer (Biorad) containing 50 $\mu$ l of 2M DTT) was added to the resin and the samples heated at 95°C for 5 minutes and then cooled on ice before 20 $\mu$ l of the mix was loaded on a SDS-PAGE gel at 0.8mA constant /cm<sup>2</sup> of gel surface for 1 hour. 25 The proteins in the gel were transferred to Immuno-Blot PVDF membranes (Bio-Rad) and probed with TCR anti  $\alpha$  chain antibody as described in Example 17 below except for the following changes. The primary antibody (TCR anti  $\alpha$  chain) and secondary antibodies were used at 1 in 200 and 1 in 1000 dilutions respectively. Figure 111 shows a picture of the developed membrane. The result shows that there is a low level 30 of TCR secretion by the yeast culture into the media.

*Example 17 - Disulphide A6 Tax TCR α and β chain expression in Baculovirus**Strategy for cloning*

The α and β chains of the disulphide A6 Tax TCR were cloned from pGMT7 into a 5 pBlueScript KS2- based vector called the pEX172. This vector was designed for cloning different MHC class II β-chains, for insect cell expression, using the leader sequence from DRB1\*0101, an AgeI site for insertion of different peptide-coding sequences, a linker region, and then MluI and SalI sites to clone the DRβ chains in front of the Jun Leucine zipper sequence. The sequence where pEX 172 differs from 10 pBlueScript II KS-, located between the KpnI and EcoRI sites of pBlueScript II KS-, is shown in Figure 112. For the purposes of cloning TCR chains in insect cells, this pEX172 was cut with AgeI and SalI to remove the linker region and MluI site, and the TCR chains go in where the peptide sequence would start. The TCR sequences were cloned from pGMT7 with a BspEI site at the 5' end (this had AgeI compatible sticky 15 ends) and a SalI site at the 3' end. In order to provide the cleavage site for the removal of the DRβ leader sequence, the first three residues of the DRβ chain (GDT) were preserved. In order to prevent the Jun Leucine zipper sequence being transcribed, it was necessary to insert a stop codon before the SalI site. For a schematic of this construct, see Figure 113. Once the TCR chains are in this plasmid, the BamHI 20 fragment were cut out and subcloned into the pAcAB3 vector, which has homology recombination sites for Baculovirus. The pAcAB3 vector has two divergent promoters, one with a BamHI site and one with a BglII cloning site. There is a BglII site in the A6 TCR β-chain, so the A6 TCR α-chain was inserted into the BglII site, and the β-chain was then subcloned into the BamHI site.

25

In accordance with the above cloning strategy, the following primers were designed (homology to the vectors is in uppercase):

A6α: F: 5' -gtagtccggagacaccggaCAGAAGGAAGTGGAGCAGAAC

30 R: 5' -gtaggtcgacTAGGAACCTTCTGGGCTGGG

A6β: F: 5' -gtagtccggagacaccggaAACGCTGGTGTCACTCAGA

R: 5' -gtaggctgacTAGTCTGCTCTACCCAGG

*PCR, cloning and sub-cloning:*

Expression plasmids containing the genes for the disulphide A6 Tax TCR  $\alpha$  or  $\beta$  chain were used as templates in the following PCR reactions. 100ng of  $\alpha$  plasmid was mixed with 1 $\mu$ l 10mM dNTP, 5 $\mu$ l 10xPfu-buffer (Stratagene), 1.25 units Pfu polymerase (Stratagene), 50pmol of the A6 $\alpha$  primers above, and the final volume was adjusted to 50 $\mu$ l with H<sub>2</sub>O. A similar reaction mixture was set up for the  $\beta$  chain, using the  $\beta$  plasmid and the pair of  $\beta$  primers. The reaction mixtures were subjected to 35 rounds of denaturation (95°C, 60 sec.), annealing (50°C, 60 sec.), and elongation (72°C, 8 min.) in a Hybaid PCR express PCR machine. The product was then digested for 2 hours at 37°C with 10 units of BspEI restriction enzyme then for a further 2 hours with 10 units of SalI (New England Biolabs). These digested reactions were ligated into pEX172 that had been digested with AgeI and SalI, and these were transformed into competent XL1-Blue bacteria and grown for 18 hours at 37°C. A single colony was picked from each of the  $\alpha$  and  $\beta$  preps and grown over night in 5 ml TYP + ampicillin (16 g/l Bacto-Tryptone, 16 g/l Yeast Extract, 5 g/l NaCl, 2.5 g/l K<sub>2</sub>HPO<sub>4</sub>, 100 mg/l Ampicillin). Plasmid DNA was purified on a QIAgen mini-prep column according to the manufacturer's instructions and the sequence was verified by automated sequencing at the sequencing facility of Genetix. The amino acid sequences of the BamHI inserts are shown in Figures 114 and 115 for the  $\alpha$  chain and  $\beta$  chain, respectively.

These  $\alpha$  and  $\beta$  disulphide A6 Tax TCR chain constructs in pEX172 were digested out for 2 hours at 37°C with BamHI restriction enzyme (New England Biolabs). The  $\alpha$  chain BamHI insert was ligated into pAcAB3 vector (Pharmingen-BD Biosciences: 21216P) that had been digested with BglII enzyme. This was transformed into competent XL1-Blue bacteria and grown for 18 hours at 37°C. A single colony was picked from this plate and grown overnight in 5 ml TYP + ampicillin and the plasmid DNA was purified as before. This plasmid was then digested with BamHI and the  $\beta$  chain BamHI insert was ligated in, transformed into competent XL1-Blue bacteria,

grown overnight, picked to TYP-ampicillin, and grown before miniprepping as before using a QIAgen mini-prep column. The correct orientation of both the  $\alpha$  and  $\beta$  chains were confirmed by sequencing using the following sequencing primers:

5 pAcAB3  $\alpha$  forwards: 5'-gaaattatgcatttggaggatg  
pAcAB3  $\beta$  forwards: 5'-attaggcctctagagatccg

*Transfection, infection, expression and analysis of A6 TCR in insect cells*

The expression plasmid containing the  $\alpha$ -chain and  $\beta$ -chain was transfected into sf9 10 cells (Pharmingen-BD Biosciences: 21300C) grown in serum free medium (Pharmingen-BD Biosciences: 551411), using the Baculogold transfection kit (Pharmingen-BD Biosciences: 21100K) as per the manufacturers instructions. After 5 days at 27°C, 200 $\mu$ l of the medium these transfected cells had been growing in was added to 100ml of High Five cells at 1x10<sup>6</sup> cells/ml in serum free medium. After a 15 further 6 days at 27°C, 1ml of this medium was removed and centrifuged at 13,000RPM in a Hereus microfuge for 5 minutes to pellet cell debris.

20 10 $\mu$ l of this insect A6 disulphide linked TCR supernatant was run alongside positive controls of bacterial A6 disulphide linked TCR 5 $\mu$ g and 10 $\mu$ g on a pre-cast 4-20% Tris/glycine gel (Invitrogen: EC60252). Reduced samples were prepared by adding 10 $\mu$ l of Reducing sample buffer (950 $\mu$ l of Laemmli sample buffer (Bio-Rad: 161-0737) 50 $\mu$ l of 2M DTT) and heating at 95°C for 5 minutes, cooling at room 25 temperature for 10 minutes then loading 20 $\mu$ l. Non-reduced samples were prepared by adding 10 $\mu$ l of Laemmli sample buffer, and loading 20 $\mu$ l.

30 The gel was run at 150 volts for 1 hour in a Novex - Xcell gel tank after which the gel was stained in 50ml of Coomassie gel stain for 1 hour with gentle agitation (1.1g Coomassie powder in 500ml of methanol stir for 1 hour add 100ml acetic acid make up to 1 litre with H<sub>2</sub>O and stir for 1 hour then filter through 0.45 $\mu$ M filter). The gel was de-stained three times for 30 mins with gentle agitation in 50ml of de-stain (as Coomassie gel stain but omitting the Coomassie powder).

Western Blots were performed by running SDS-PAGE gels as before but the proteins were transferred to Immuno-Blot PVDF membranes (Bio-Rad: 162-0174) rather than staining the gels with Coomassie. Six filter papers were cut to the size of the gel and 5 soaked in transfer buffer (2.39g Glycine, 5.81g of Tris Base, 0.77g DTT dissolved in 500mls of H<sub>2</sub>O, 200mls of methanol added then made up to 1000mls with H<sub>2</sub>O). The PVDF membrane was prepared by soaking in methanol for 1 minute and then in transfer buffer for 2 minutes. Three filter papers were placed on the anode surface of the Immuno-blot apparatus (Pharmacia – Novablot) then the membrane was placed on 10 top followed by the gel and then finally three more filter papers on the cathode side. The Immuno-blot was run for 1 hour at 0.8mA constant /cm<sup>2</sup> of gel surface.

After blotting, the membrane was blocked in 7.5mls of blocking buffer (4 Tris-buffered saline tablets (Sigma: T5030), 3g non-fat dried milk (Sigma: M7409), 30μl of 15 Tween 20 made up to 30mls with H<sub>2</sub>O) for 60 mins with gentle agitation. The membrane was washed three times for 5 mins with TBS wash buffer (20 TBS tablets, 150μl Tween 20 made up to 300ml with H<sub>2</sub>O). The membrane was then incubated in primary antibody 1 in 50 dilution of anti TCR α chain clone 3A8 (Serotec: MCA987) or anti TCR β chain clone 8A3 (Serotec: MCA988) in 7.5ml blocking buffer for 1 20 hour with gentle agitation. The membrane was washed as before in TBS wash buffer. Next, a secondary antibody incubation of HRP labelled goat anti-mouse antibody (Santa Cruz Biotech: Sc-2005) 1 in 1000 dilution in 7.5ml of blocking buffer was carried out for 30 min with gentle agitation. The membrane was washed as before and then washed in 30ml of H<sub>2</sub>O with 2 TBS tablets.

25 The antibody binding was detected by Opti-4CN colourmetric detection (Biorad: 170-8235) (1.4ml Opti-4CN diluent, 12.6ml H<sub>2</sub>O, 0.28ml Opti-4CN substrate). The membranes were coloured for 30 minutes and then washed in H<sub>2</sub>O for 15 minutes. The membranes were dried at room temperature, and scanned images were aligned 30 with an image of the coomassie stained gel (Figure 116).

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*Results*

It can be seen from Figure 116 that both disulphide TCRs are formed as a heterodimer that is stable in the SDS gel. They both break into the  $\alpha$  and  $\beta$  chains upon reduction. The insect disulphide TCR heterodimer has a slightly higher molecular weight than the 5 bacterially produced version, presumably because of the glycosylation from the insect cells. It can be seen that in this instance the insect cells are producing  $\alpha$  chain in excess, and free  $\alpha$  chain can be seen in the non-reduced lane of the anti- $\alpha$  western blot.

10 These data clearly demonstrate that the baculovirus expression system described above provides a viable alternative to prokaryotic expression of soluble TCRs containing novel disulphide bonds.

**Claims**

1. A soluble T cell receptor (sTCR), which comprises (i) all or part of a TCR  $\alpha$  chain, except the transmembrane domain thereof, and (ii) all or part of a TCR  $\beta$  chain, except the transmembrane domain thereof, wherein (i) and (ii) each comprise a functional variable domain and at least a part of the constant domain of the TCR chain, and are linked by a disulphide bond between constant domain residues which is not present in native TCR.
- 10 2. A sTCR as claimed in claim 1, wherein one or both of (i) and (ii) comprise all of the extracellular constant Ig domain of the TCR chain.
- 15 3. A sTCR as claimed in claim 1 or claim 2, wherein one or both of (i) and (ii) comprise all of the extracellular domain of the TCR chain.
4. A soluble  $\alpha\beta$ -form T cell receptor (sTCR), wherein a covalent disulphide bond links a residue of the immunoglobulin region of the constant domain of the  $\alpha$  chain to a residue of the immunoglobulin region of the constant domain of the  $\beta$  chain.
- 20 5. A sTCR as claimed in any preceding claim, wherein an interchain disulphide bond in native TCR is not present.
- 25 6. A sTCR as claimed in claim 5, wherein native  $\alpha$  and  $\beta$  TCR chains are truncated at the C-terminus such that the cysteine residues which form the native interchain disulphide bond are excluded.
7. A sTCR as claimed in claim 5, wherein cysteine residues which form the native interchain disulphide bond are substituted to another residue.
- 30 8. A sTCR as claimed in claim 7, wherein cysteine residues which form the native interchain disulphide bond are substituted to serine or alanine.

9. A sTCR as claimed in any preceding claim, wherein an unpaired cysteine residue present in native TCR  $\beta$  chain is not present.
10. A sTCR as claimed in any preceding claim, wherein the disulphide bond which is not present in native TCR is between cysteine residues substituted for residues whose  $\beta$  carbon atoms are less than 0.6 nm apart in the native TCR structure.  
5
11. A sTCR as claimed in any preceding claim, wherein the disulphide bond which is not present in native TCR is between cysteine residues substituted for Thr 48 of exon 1 of TRAC\*01 and Ser 57 of exon 1 of TRBC1\*01 or TRBC2\*01.  
10
12. A sTCR as claimed in any one of claims 1 to 10, wherein the disulphide bond which is not present in native TCR is between cysteine residues substituted for Thr 45 of exon 1 of TRAC\*01 and Ser 77 of exon 1 of TRBC1\*01 or TRBC2\*01.  
15
13. A sTCR as claimed in any one of claims 1 to 10, wherein the disulphide bond which is not present in native TCR is between cysteine residues substituted for Tyr 10 of exon 1 of TRAC\*01 and Ser 17 of exon 1 of TRBC1\*01 or TRBC2\*01.
- 20 14. A sTCR as claimed in any one of claims 1 to 10, wherein the disulphide bond which is not present in native TCR is between cysteine residues substituted for Thr 45 of exon 1 of TRAC\*01 and Asp 59 of exon 1 of TRBC1\*01 or TRBC2\*01.
15. A sTCR as claimed in any one of claims 1 to 10, wherein the disulphide bond which is not present in native TCR is between cysteine residues substituted for Ser 15 of exon 1 of TRAC\*01 and Glu 15 of exon 1 of TRBC1\*01 or TRBC2\*01.  
25
16. A sTCR as claimed in any one of claims 1, 2 and 5 to 15, wherein (i) and (ii) each comprise the functional variable domain of a first TCR fused to all or part of the constant domain of a second TCR, the first and second TCRs being from the same species.  
30

17. A sTCR as claimed in claim 16, wherein the constant domains of the second TCR are truncated N-terminal to the residues which form the non-native interchain disulphide bond.
- 5 18. A sTCR as claimed in any preceding claim, wherein one or both of the chains are derivatised with, or fused to, a moiety at its C or N terminus.
- 10 19. A sTCR as claimed in any preceding claim, wherein one or both of the chains have a cysteine residue at its C and/or N terminus to which a moiety can be fused.
20. A sTCR as claimed in any preceding claim, further comprising a detectable label.
- 15 21. A sTCR as claimed in any preceding claim associated with a therapeutic agent.
22. A multivalent T cell receptor (TCR) complex comprising a plurality of sTCRs as claimed in any preceding claim.
- 20 23. A complex as claimed in claim 22, comprising a sTCR multimer.
24. A complex as claimed in claim 23, comprising two or three or four or more T cell receptor molecules associated with one another, preferably via a linker molecule
- 25 25. A complex as claimed in claim 22, 23 or 24, wherein the sTCRs or sTCR multimers are present in a lipid bilayer or are attached to a particle.
26. A method for detecting MHC-peptide complexes, which comprises:
  - (i) providing a soluble TCR as claimed in any one of claims 1 to 21 or a multivalent T cell receptor complex as claimed in any one of claims 22 to 25;
  - 30 (ii) contacting the soluble TCR or multivalent TCR complex with the MHC-peptide complexes; and

(iii) detecting binding of the soluble TCR or multivalent TCR complex to the MHC-peptide complexes.

27. A pharmaceutical formulation comprising a sTCR as claimed in any one of 5 claims 1 to 21, and/or a multivalent TCR complex as claimed in any one of claims 22 to 25, together with a pharmaceutically acceptable carrier.

28. A nucleic acid molecule comprising a sequence encoding (i) or (ii) of a sTCR as claimed in any one of claims 1 to 21, or a sequence complementary thereto.

10 29. A vector comprising a nucleic acid molecule as claimed in claim 28.

30. A host cell comprising a vector as claimed in claim 29.

15 31. A method for obtaining (i) or (ii) as defined in any one of claims 1 to 21, which method comprises incubating a host cell as claimed in claim 30 under conditions causing expression of the peptide and then purifying the polypeptide.

20 32. A method as claimed in claim 31, further comprising mixing (i) and (ii) under suitable refolding conditions.